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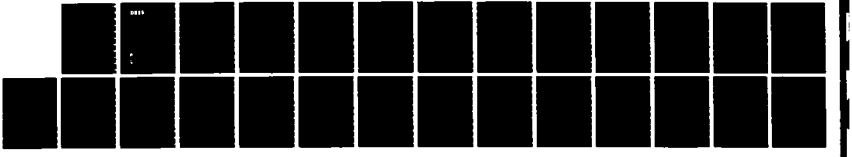

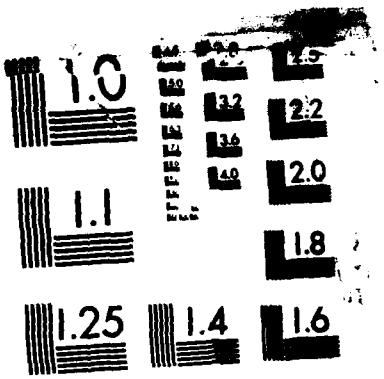

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NO. 1166

MEASUREMENT OF MACROPHAGE ACTIVATION BY CHEMILUMINESCENCE (U)

by

R. Lippé*, R.J.F. Markham and N.P. Erhardt

Project No. 16A10

May 1986

* Summer Research Assistant, University of Montreal, May 1985 — September 1985

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ABSTRACT

Contd from pg 1

Chemiluminescence (CL) stimulated by the phagocytosis of yeast particles was used to monitor metabolic activity of murine macrophages obtained by either peritoneal or bronchoalveolar lavage. Intraperitoneal injection of the macrophage activator lipopolysaccharide (LPS) from Escherichia coli led to the appearance of macrophages in the peritoneal lavage fluid which had enhanced CL when compared to macrophages from control animals injected with saline or phosphate buffered saline. Intraperitoneal injection of LPS, however, was not able to enhance the CL activity of cells obtained by bronchoalveolar lavage whereas intratracheal instillation of LPS directly into the lung did elicit a population of cells with increased chemiluminescence. Thus it appears that CL is a useful measure of macrophage activation. The appropriateness of the CL technique was substantiated by the findings that enhanced CL induced by LPS is correlated to enhanced oxidative metabolism as measured by an increased production of hydrogen peroxide.

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INTRODUCTION

Because the lung is exposed to a vast number of airborne pathogens, pulmonary infections are common. For this reason, the lung is equipped with an array of mechanisms to protect itself from this challenge (1). In the lower lung, two major mechanisms are responsible for clearance of organisms reaching the alveolar spaces; the specific immune system and a non-specific phagocytic system. Stimulation of the immune system by an organism in the form of a vaccine can provide effective protection against virulent challenge but only against that particular agent. Therefore, in order to obtain protection from all possible pathogens, the lung must be exposed to and respond specifically to each different agent. The non-specific phagocytic system, however, responds to most organisms in a similar manner and thus will be active against a far broader range of potential pathogens.

Enhancement, or activation of the non-specific aspects of pulmonary

defense would thus appear to a suitable approach for protection against pulmonary infection as it would emcompass a greater number of potential pathogens. The alveolar macrophage, the predominant phagocytic cell in the distal lung, would be a target of this activation (2). There are a variety of compounds which have the potential to enhance non-specific clearance of pathogens from the lung by virtue of their ability to activate alveolar macrophages (3,4,5). The first step in determining the efficacy of these compounds is to devise techniques to accurately measure the functional capacity of the alveolar macrophage. A recently described technique, chemiluminescence (CL), appears to be a reliable method to measure the activity of macrophages (6). The test is based upon release of reactive oxygen species during phagocytosis of particles or upon stimulation of macrophages with soluble activators like phorbol myristate acetate (PMA). This oxidative metabolism can be intensified by exposure to certain bacterial compounds, products of activated lymphocytes or some synthetic materials (6,7,8).

To initially assess CL as a measure of macrophage activation, peritoneal macrophages were chosen because of their abundance and ease of collection. A known macrophage activator, lipopolysaccharide (LPS) from Escherichia coli, was used to activate the cells in vivo.

MATERIALS AND METHODS

Animals: Male C57BL/HPB mice aged 4-7 weeks were obtained from the University of Calgary. For each experiment, mice were matched by age and weight. They were housed and cared for in a manner consistant with the guidelines set by the Canadian Council on Animal Care.

Inoculations: Intraperitoneal injections were performed using a 27 1/2 g needle with volumes up to 1.0 ml. Stock solutions of LPS from E. coli

0128:B12 (Sigma Chemicals Co., St. Louis, MO) were diluted in sterile phosphate buffered saline (PBS: Dulbecco A, Oxoid, England) or saline prior to injection. Controls received saline or PBS. Intratracheal inoculations were performed on anesthetized mice. Animals were given sodium pentobarbitone at a dosage of 0.07 mg/gm body weight. The neck region was swabbed with 70% ethanol and an incision was made to expose the trachea. A 27 1/2 gauge needle was bent to approximately 45° and connected to a 1 ml syringe filled with LPS or saline. The needle was inserted into the trachea and solution was injected. The skin was closed with surgical staples and mice were placed in an incubator at 37°C until recovery. Those that recovered from anesthesia appeared well. There was no later indication of infectious disease due to surgical manipulation.

Macrophages: Macrophages were obtained by either peritoneal lavage with PBS as described by Mishell & Shiigi (9) or by bronchoalveolar lavage. Briefly mice were sacrificed by CO_2 inhalation and an incision was made to expose the trachea. The trachea was then partially severed and a 21 g needle was inserted and tied in place with a surgical suture. PBS was alternatively instilled and retrieved from the lung until approximately 4-5 mL of fluid was recovered from these lavages. A similar volume was collected from the peritoneal lavages. A 500 ul aliquot of the suspensions was cytocentrifuged (Shandon Cytospin 2) and slides were stained with Diff-Quik® (American Scientific Products, McGaw Park, IL) to determine if purification of cells was needed. If purification was indicated by the presence of >10% polymorphonuclear leukocytes (PMN), separation of cellular populations was accomplished using a Ficoll-Hypaque (F/H) gradient (Ficoll-Paque Pharmacia Fine Chemicals, Piscataway N.J.). Ten to twelve milliliters of the cell suspension were layered on a cushion of 3-4 mL F/H and centrifuged at 675 x g for 30 minutes. Mononuclear cells including macrophages were collected from the F/H-PBS interface and were washed with 12 mL of PBS by centrifugation for 15 minutes at 400 x g. The pellet was then resuspended in PBS. If F/H separation was not necessary, cells were washed once as above. Cell

suspensions were counted using an automated cell counter (Sysmex model CC-110) and the mononuclear cells were resuspended to 3.0 x 10^6 cells/mL in PBS. All cells were kept on ice from the time of the lavage until the assay was performed except for centrifugation which was carried out at room temperature.

Preparation of zymosan: One hundred and fifty milligrams of zymosan from Saccharomyces cerevisiae (Sigma) was suspended in 30 mL saline and boiled for 30 minutes. Ten milliliters of this suspension was centrifuged for 10-15 minutes at 300 x g. Fluid was removed and the pellet was incubated with 2-3 mL normal mouse serum at 37° for 30 minutes. The suspension was centrifuged for 10-15 min. at 300 x g and the pellet was resuspended to 1 mg/mL in medium RPMI 1640 (Flow Lab., McClean VA.) or Minimal Essential Medium (MEM, Flow Lab.). Zymosan was kept frozen at -70°C until used.

Chemiluminescence assay: Unless otherwise specified, 400 uL of a 3.0 x 10^6 cells/mL suspension was used for assaying both peritoneal and alveolar cells. Fifteen or 20 uL of 2 x 10^{-3} M lucigenin in RPMI and 100 ul or 200 ul opsonized zymosan was used for peritoneal or alveolar cells respectively. All reagents were kept at -70°C until use. A Packard Pico-Lite Luminometer (Model 6100) was used to detect chemiluminescence, the measure of which was counts per minute (cpm).

Cells and lucigenin were added to the vials and background counts were measured. When these counts had stablilized, zymosan was added with a Hamilton syringe. Addition of this reagent determined time zero for the assay. These suspensions were mixed with the syringe and readings taken 0, 5 and 10 min. and at subsequent 10 min. intervals.

Measurement of Hydrogen peroxide. The colorimetric method of Pick and Keisari (10) was used to detect hydrogen peroxide secreted by macrophages. It is based upon a change in the spectral curve of phenol red subsequent to peroxidase-mediated oxidation of phenol red by hydrogen peroxide. Three

million cells were incubated with 20 nM Phorbol Myristate Acetate (PMA) to stimulate oxidative metabolism.

RESULTS

Variation among mice: In order to determine the variation in the individual responses, we compared the CL response from 4 mice injected intraperitoneally with 5 ug of LPS. The assay was performed 48 hours later. The average CL value was 243,383 cpm and the standard deviation was 94,319 (Fig. 1).

When cells from 2-3 mice were pooled prior to the assay, it was possible to decrease this variation. Samples from 3 separate pools showed a decreased variation (Fig. 2) with only 7% variation when peaks responses were compared. Of that 7%, we determined that 3% was due to counting error in the luminometer itself. All subsequent experiments used pools of at least three mice per trial.

Chemiluminescence as a measure of macrophage activity: Assays performed with unelicited peritoneal cells and LPS-elicited cells showed an augmentation of CL counts of at least 45% (Fig. 3) in all the LPS treated groups. Measurement of hydrogen peroxide generation (Table 1) confirmed the activating effect of LPS. Figure 3 also demonstrates that greatest activation occurred 48 hrs after injection of LPS.

Effect of PBS volume in the injections: Assays performed at 72 hours showed that the peak responses are affected by the volume used for injecting LPS or PBS control or saline. (Fig. 4). Increasing amounts of saline appeared to increase the CL response. Therefore, in all subsequent experiment, the volume injected into both control and test animals was kept to a minimal amount (0.1 ml).

Effect of the LPS dose: Peritoneal cells recovered at 48 hours after injection of different amounts of LPS showed that chemiluminescence is related to the dose of LPS. Highest CL values occurred with 0.1 ug of LPS; 29% higher than the next highest peak (1.0 ug) and more than 300% higher than the control (Fig. 5).

Cell morphology: Stained slides were made not only to determine if purification of cells was required but also to observe morphologic changes which had occured during stimulation with LPS. Activated macrophages generally appeared larger than the non-activated ones and contained larger and/or more vacuoles.

Another effect of the LPS treatment was a variation in the types of cells recovered in the lavages. Slides made at 24 and 48 hours after the injections revealed an increase in the number of PMNs. The PMNs often represented the majority of the cells at 24 hours post-injection, whereas in control or unelicited populations macrophages were usually the most common type of cell. By 48-72 hours post-inoculation, the proportions returned to those observed in cells from control animals.

These alterations in cell populations were seen both in peritoneal and alveolar cells when injections were made locally (I.P. and I.T. respectively). Alveolar cells from mice receiving an I.P. injection of LPS did not show any changes when compared to those from control animals.

LPS activation of the alveolar macrophages: Intraperitoneal injections of LPS did not result in a population of alveolar macrophages that gave an enhanced CL response (Fig. 6). Intratracheal inoculation, however, was able to induce an increase in the CL response in cells recovered in the lavage (Figure 7).

DISCUSSION

The capability of LPS to activate or enhance the metabolic and functional characteristics of macrophages and other leukocytes has been reported. LPS has been shown to non-specifically activate or enhance phagocytosis, cytotoxicity and oxidative metabolism including those reactions responsible for chemiluminescence (11-13). This activation has been correlated with an increased ability of these phagocytes to kill engulfed bacteria or other organisms and an enhanced protection against challenge with virulent infectious agents *in vivo* (14,15). Data presented in this communication shows that CL can be used directly as a measure of macrophage activation by LPS. LPS has previously been shown to enhance superoxide anion and hydrogen peroxide production by phagocytes (16,17), but to our knowledge this is the first report of LPS enhancing CL directly. This finding is supported by data showing enhanced CL following activation by other bacterial products like muramyl dipeptide or cell walls of *Corynebacterium parvum* (18, 19).

CL is the result of generation of reactive oxygen species by a membrane associated NADPH oxidase present in mononuclear phagocytes and neutrophils. This occurs during phagocytosis or perturbation of the cell membrane by soluble products. In order to assay this event, enhancers of chemiluminescence such as lucigenin and luminol are required to amplify the reaction. Enhancement of CL by lucigenin appears to involve superoxide anion itself generated directly by the membrane-bound oxidase while luminol enhancement appears to require products associated with lysosomal myeloperoxidase making this latter compound not suitable for studying CL in myeloperoxidase-poor macrophages. Generation of hydrogen peroxidase by action of superoxide dismutase is also reflective of oxidative events associated with phagocytosis and our finding of enhanced H_2O_2 production in LPS-treated animals confirms activation of oxidative metabolism in LPS-treated mice.

The appearance of PMNs in the peritoneum and lungs of LPS treated mice may also be indicative of enhanced macrophage activity. Macrophages have been shown to release factors which can attract neutrophils to inflammatory sites, particularily into the lungs (20). It is likely, however, that local phlogistic mediators generated from certain complement components by LPS are also involved in the attraction of the PMNs to these sites independent of the macrophages (20).

The effect of LPS is related to the time between administration and collection of the cells with maximal stimulation of CL occurring 48 hours after administration of LPS with substantial reduction in effect taking place after that maximal value has been obtained. If the intent of administration of these drugs is to activate cells so that they can better withstand bacterial infection, it may be necessary to tailor the type and administration regimen of the drugs to speed up and prolong the state of activation. We have not challenged LPS treated mice with infectious agents to determine whether this enhanced CL activity relates to protection in our system. This measure of the host's response (i.e. protection) may be the eventual determinant of choice of drug and treatment protocol.

Intraperitoneal injections of LPS do not activate alveolar macrophages. The LPS is probably eliminated before it can reach the lung in a sufficient amount to give a discernable effect. It has been reported that LPS is rapidly bound to cells and proteins in the blood following intravenous injection (21) and hence routes other that direct inoculation of LPS into the lung may not provide enough material to the target organ. That contention is supported by the fact that I.T. inoculation of LPS does activate the alveolar macrophages in terms of CL counts and presence of PMNs; thus if LPS reaches the lungs it can activate the cells.

While LPS is capable of activating peritoneal and alveolar macrophages in situ, it is an unlikely candidate for use in human or animal trials owing to its known toxic effects. Other candidates for macrophage activation which are more appropriate may be now chosen using CL as a primary screening assay. Should the drug of choice possess the ability to activate alveolar macrophages, it could then be utilized in a challenge system to determine if it could afford protection from respiratory challenge with an infectious agent.

CONCLUSION

Pooling cells reduces the variation observed among the mice from 39% to 7%. Using these pooled cells, we are able to establish chemiluminescence as a measure of macrophage activity and of activation of these cells with LPS.

Intraperitoneal injections of LPS activate the peritoneal macrophages whereas I.T. injections activate the alveolar macrophages. Activation is related to the dose of LPS as well as the delay between the injections and the CL assay. Maximal results are obtained with 0.1 ug of LPS given 48 hours prior to assay. Intraperitoneal inoculations of LPS failed to activate the alveolar cells likely due to insufficient LPS reaching the lung. However, alveolar macrophages were activated when the LPS was delivered directly in the lungs.

REFERENCES

1. Green, G.M., Jakab, G.J., Low, R.B., Davis, G.S. Defense mechanisms of the respiratory membrane. Am Rev Resp Dis 115:479-510 1977

2. Hocking, W.G., Golde, D.W. The pulmonary-alveolar macrophage. New Eng J Med 301:580-644 1979.

3. Loveless, S.E., Munson, A.E. Maleic vinyl ether activation of murine macrophages against lung metastasizing tumors. Cancer Res 41:3901-3906 1981

4. Fidler I.J., Sone, S., Fogler, W.E., Barnes, Z.L. Eradication of spontaneous metastases and activation of alveolar macrophages by intravenous injection of liposomes containing muramyl dipeptide. Proc Nat Acad SCi 78:1680-1684 1981

5. Sone, S., Mutsuura, S., Ogawara, M., Utsugi, T., Tsubura, E. Activation by a new acyltripeptide and its analogs in liposomes of rat alveolar macrophages to the tumor cytotoxic state. Cancer Immunol Immunother 18:169-173 1984

6. Schleupner, C.J., Glasgow, L.A. Peritoneal macrophage activation indicated by enhanced chemiluminescence. Infect Immun 21:886-895 1978

7. Pabst, M.J., Johnson, R.B. Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or lipopolysaccharide. J Exp Med 151:101-114 1980.

8. Johnston, R.B. Enhancement of phagocytosis associated oxidative metabolism as a manifestation of macrophage activation. Lympokines 3:33-55 1981

9. Mishell, B.B., Shiigi, S.M. Selected Methods in Cellular Immunology. San Francisco, W.H. Freeman and Company. 1980.

10. Pick, E., Keisari, Y. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Meth 38:161-170 1980

11. Lokesh, B.R., Wrann, M. Lipopolysaccharide significantly enhances erythrophagocytosis but marginally stimulates the phagocytosis of Staphylococcus aureus in mouse peritoneal macrophages. Immunobiol 169:11-20 1985

12. Pace, J.L., Russell, S.W. Activation of mouse macrophages for tumor cell killing. Quantitative analysis of the interactions between lymphokine and lipopolysaccharide. J. Immunol 126:1863-1867 1981

13. Kuro, M., Yagawa, K., Nagao, S., Tanaka, A. Enhanced superoxide anion release from phagocytes by muramyl dipeptide and lipopolysaccharide. Infect Immun 39:559-564 1983

14. Sasada, M., Johnston, R.B. Macrophage microbicidal activity. Correlation between phagocytosis associated oxidative metabolism and the killing of landida by macrophages. J Exp Med 152:85-98 1980

I5. Osada, Y., Mitsuyama, M., Matsumoto, K., Une, T., Otani, T., Ogawa, H., Nomoto, K. Stimulation of resistance of immunocompromised mice by a muramyl lipeptide analog. Infect Immun 37:1285-1288 1982

16. Kaku, M., Yagawa, K., Nagao, S., Tanaka, A. Enhanced superoxide anion release from phagocytes by muramyl dipeptide or lipopolysaccharide. Infect Immun 39:559-564 1983

17. Guthrie, L.A., McPhail, L.C., Henson, P.M., Johnston, R.B. Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. J Exp Med 160:1656-1671 1984

18. Bryant, S.M. Enhanced chemiluminescence production by purified macrophages monolayers from C. parvum-treated mice. Immunopharmacol 3:19-29 2981

19. Mashihi, K.N., Azuma, I., Brehmer, W., Lange, W. Stimulation of chemiluminescence by synthetic muramyl dipeptide and analogs. Infect Immun. 40:16-21 1983

20. Slauson D.O. The mediation of pulmonary inflammatory injury. Adv in Vet Sci and Comp Med 26:99-153 1982

21. Wilson, M.E. Effects of bacterial endotoxins on neutrophil function. Rev Infect Dis 7:404-418 1985

Table 1: Production of Hydrogen Peroxide by Murine Peritoneal Macrophages Stimulated by Lipopolysaccharide.

Treatment[+]	nM Hydrogen Peroxide/mg cellular protein[++]
Saline	51 ± 23*
LPS	107 ± 10

[+] Mice injected 72 hours previously with saline or 1.0 μg LPS

[++]Amount of hydrogen peroxide produced by 3 x 10^6 cells 2 hours after stimulation with PMA

* ± one standard deviation. LPS group was significantly greater than control ($p<0.01$) as measured by Student's T-test.

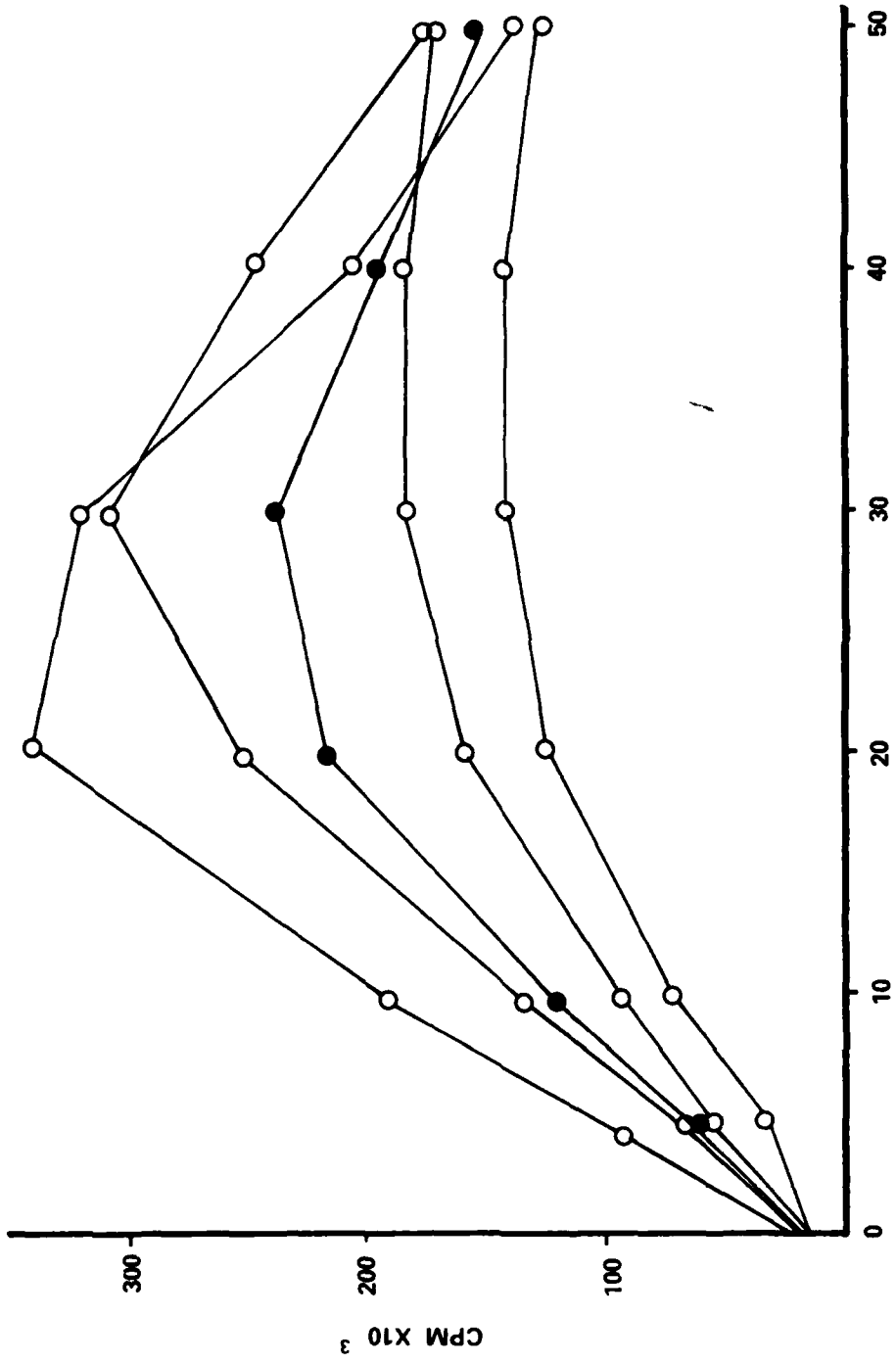

FIGURE 1

Chemiluminescence of peritoneal exudate cells lavaged from mice injected 48 hours previously with 5 μg LPS given intraperitoneally. Mean of the peak responses was 243,383 ± 94,319. Open circles represent individual mice. The closed circle represents the mean response at the given time intervals.

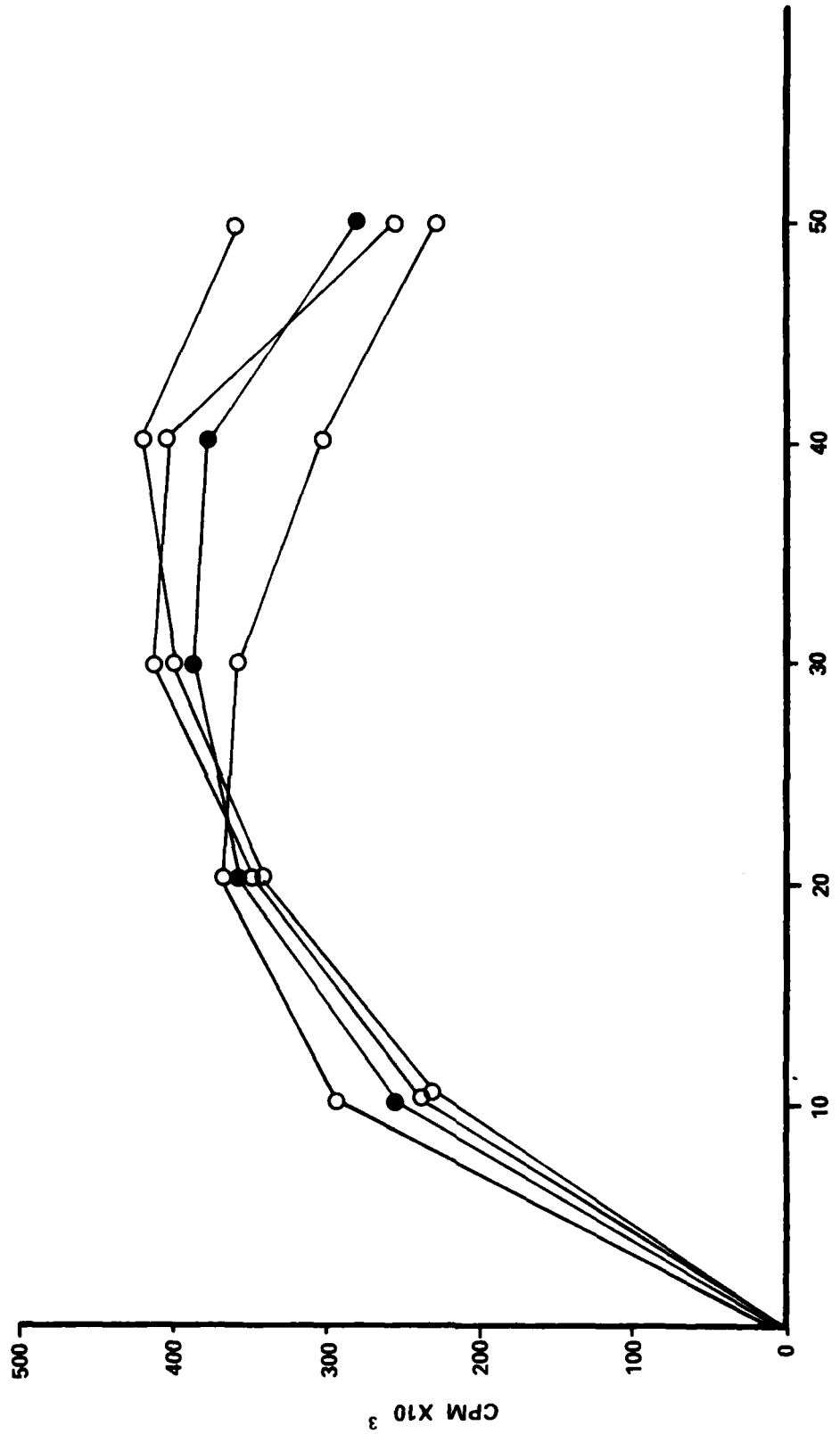

FIGURE 2

Chemiluminescence of peritoneal exudate cells lavaged from mice injected 48 hours previously with 5 μg LPS given intraperitoneally. Mean of the peak responses was 400,305 ± 29,576. Open circles represent pools of 2-3 mice. The closed circle represents the mean response of the 3 pools at the given time intervals.

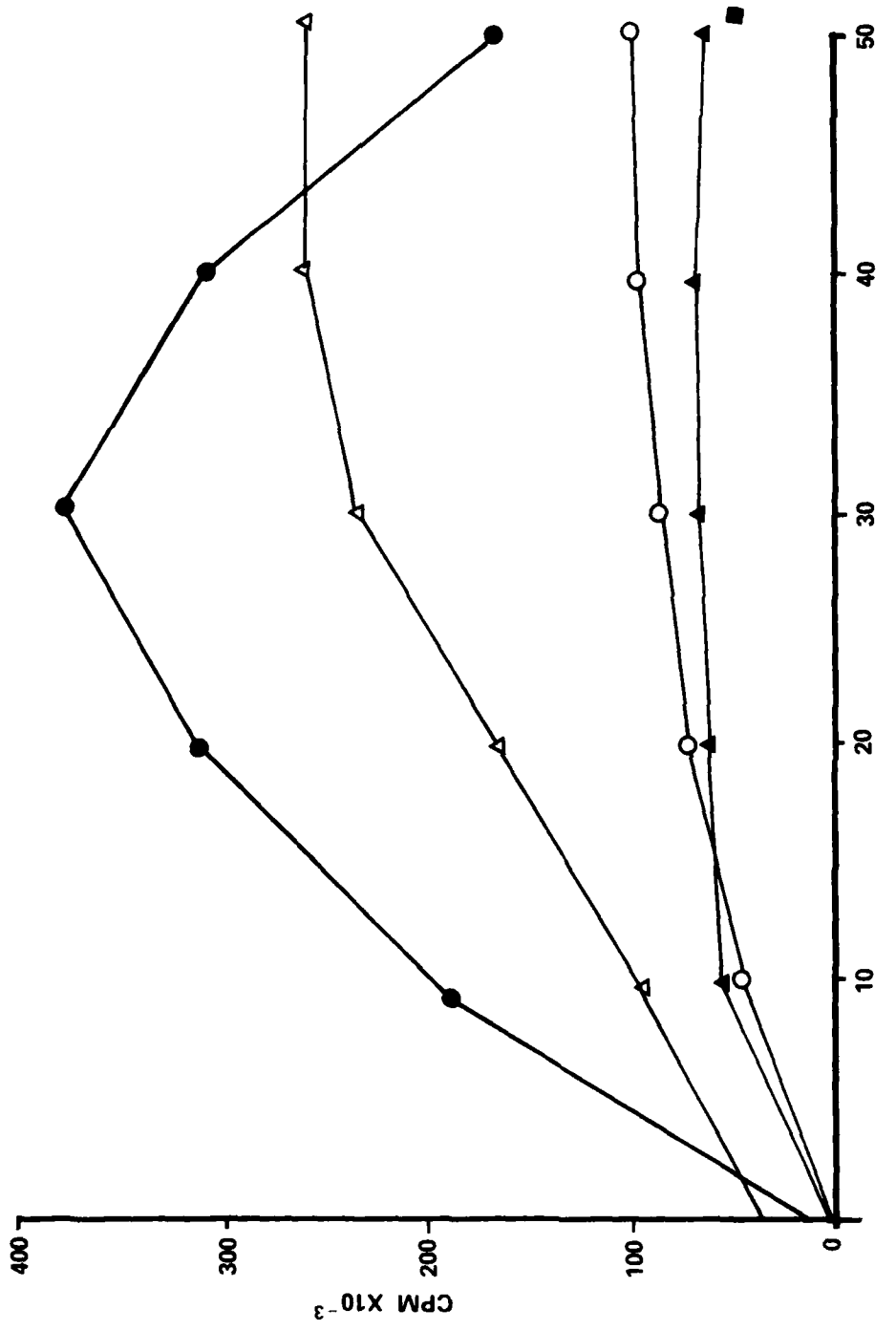

FIGURE 3

Time course of chemiluminescence of peritoneal exudate cells lavaged from mice at different time periods after intraperitoneal injection of 5 μg LPS. ○—○ 24 hours; ●—● 48 hours; △—△ 96 hours; ▲—▲ control cells from uninjected animals.

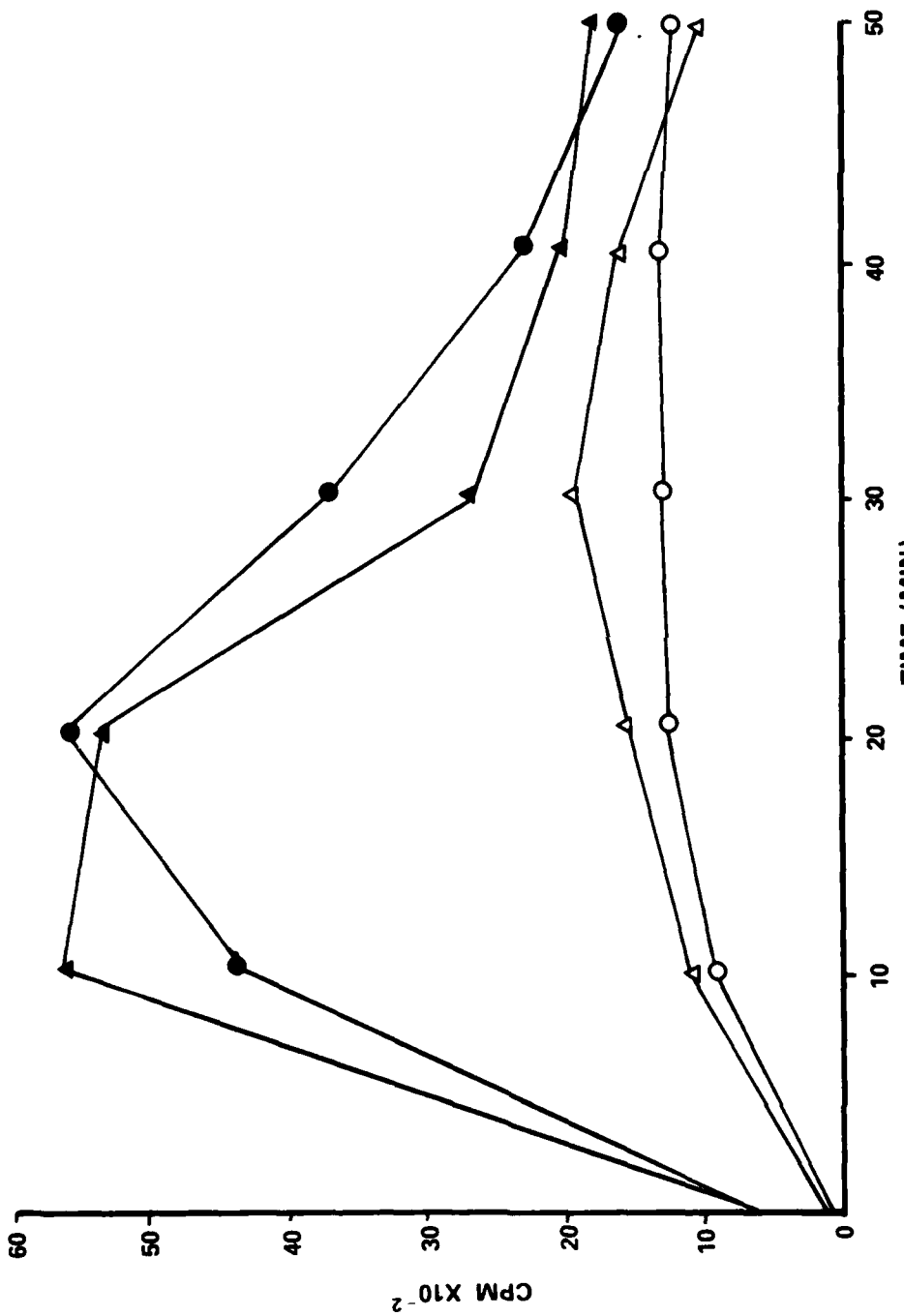

FIGURE 4

Effect of the volume of inoculation on the chemiluminescence of peritoneal exudate cells lavaged from mice injected 72 hours after intraperitoneal injection of PBS or LPS: ○—○ 0.1 mL PBS; ●—● 0.1 mL LPS (0.01 μg); △—△ 0.05 mL PBS; ▲—▲ 0.05 mL LPS (0.5 μg).

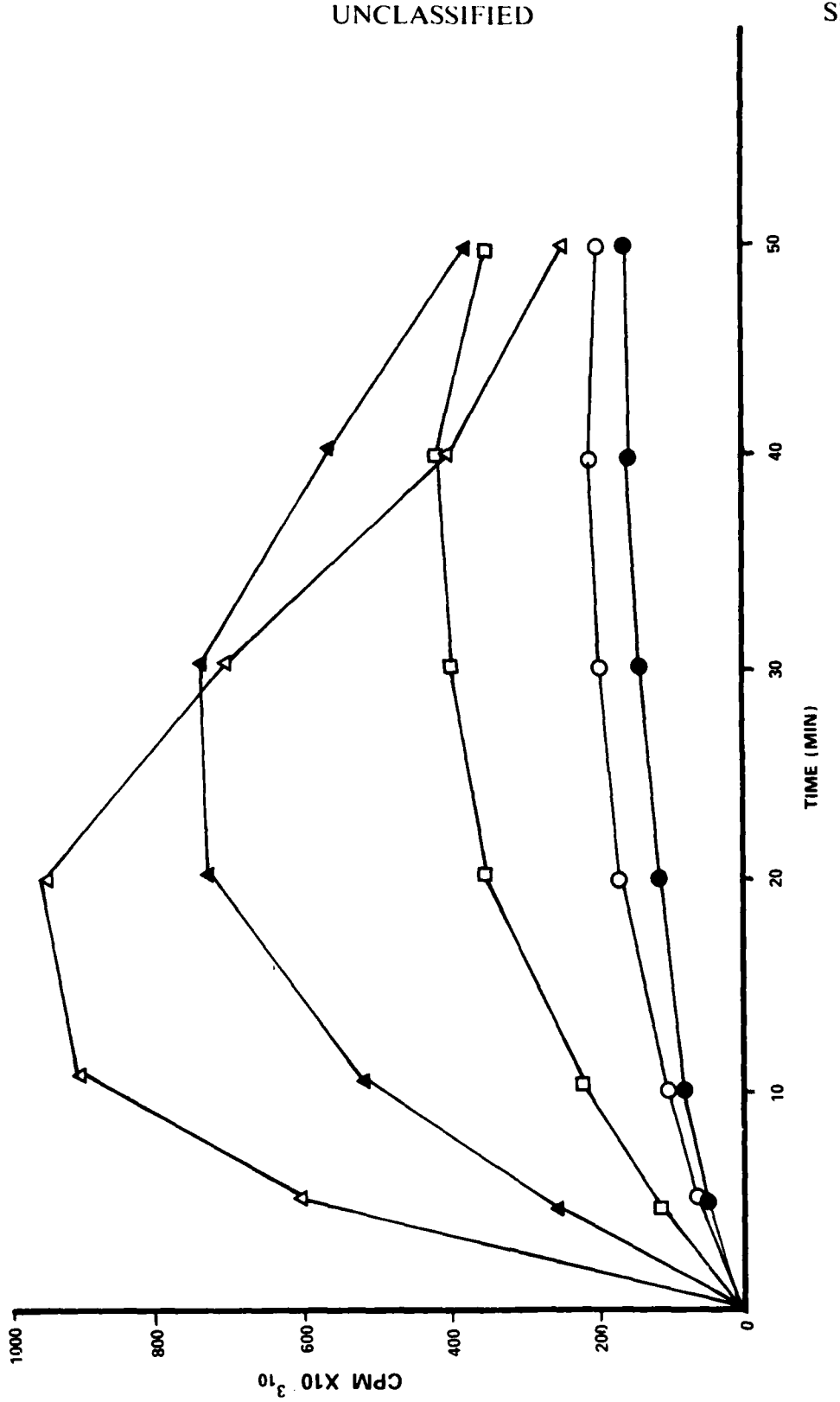

FIGURE 5

Effect of dose of LPS on the chemiluminescence of peritoneal exudate cells lavaged from mice 48 hours after intraperitoneal injection of LPS. ○—○ control PBS; ●—● 0.01 μg; △—△ 0.1 μg; ▲—▲ 1.0 μg; □—□ 10.0 μg.

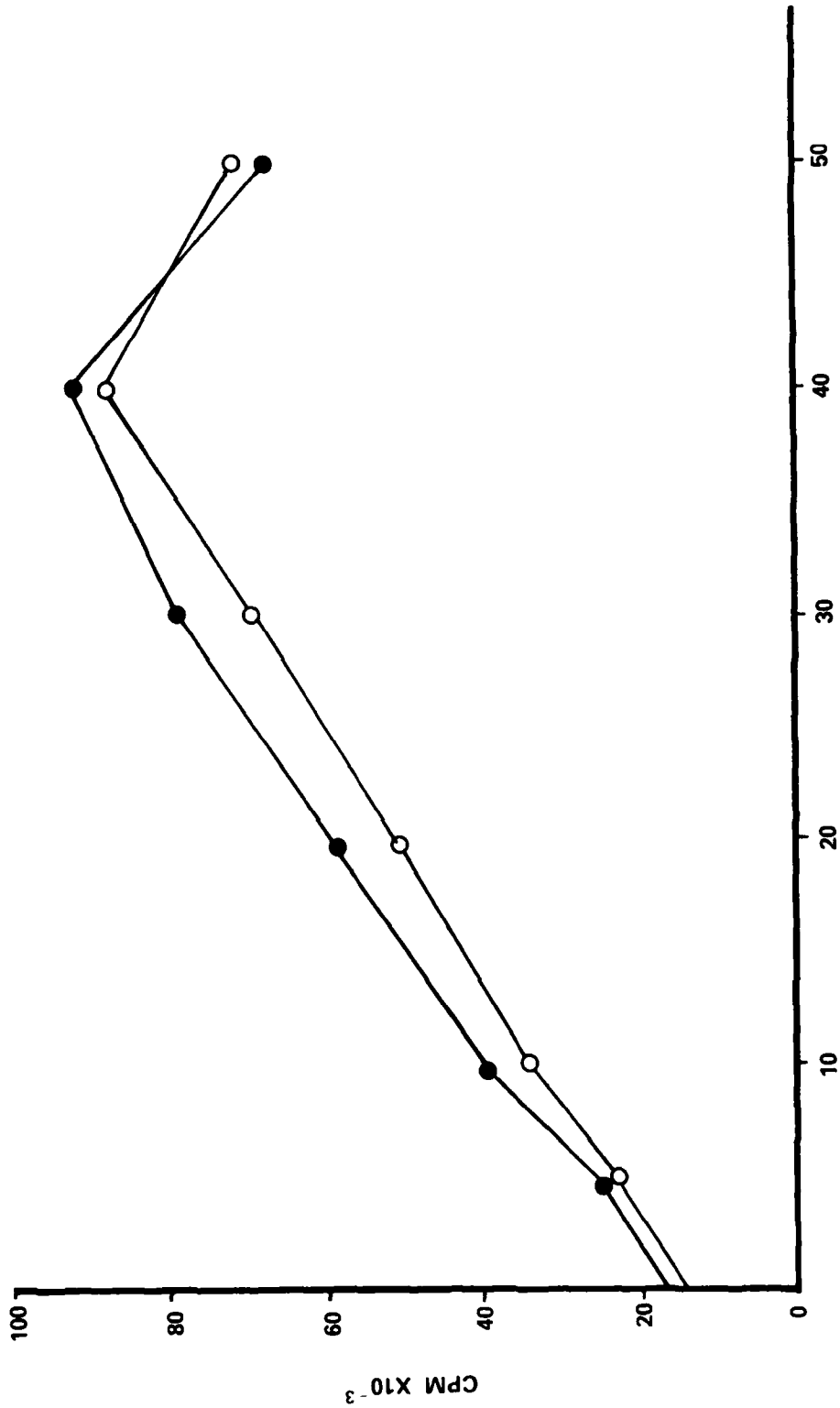

FIGURE 6

Chemiluminescence of bronchoalveolar cells lavaged from mice 48 hours after intraperitoneal injection of 5 μg LPS. ○—○ control (PBS); ●—● LPS.

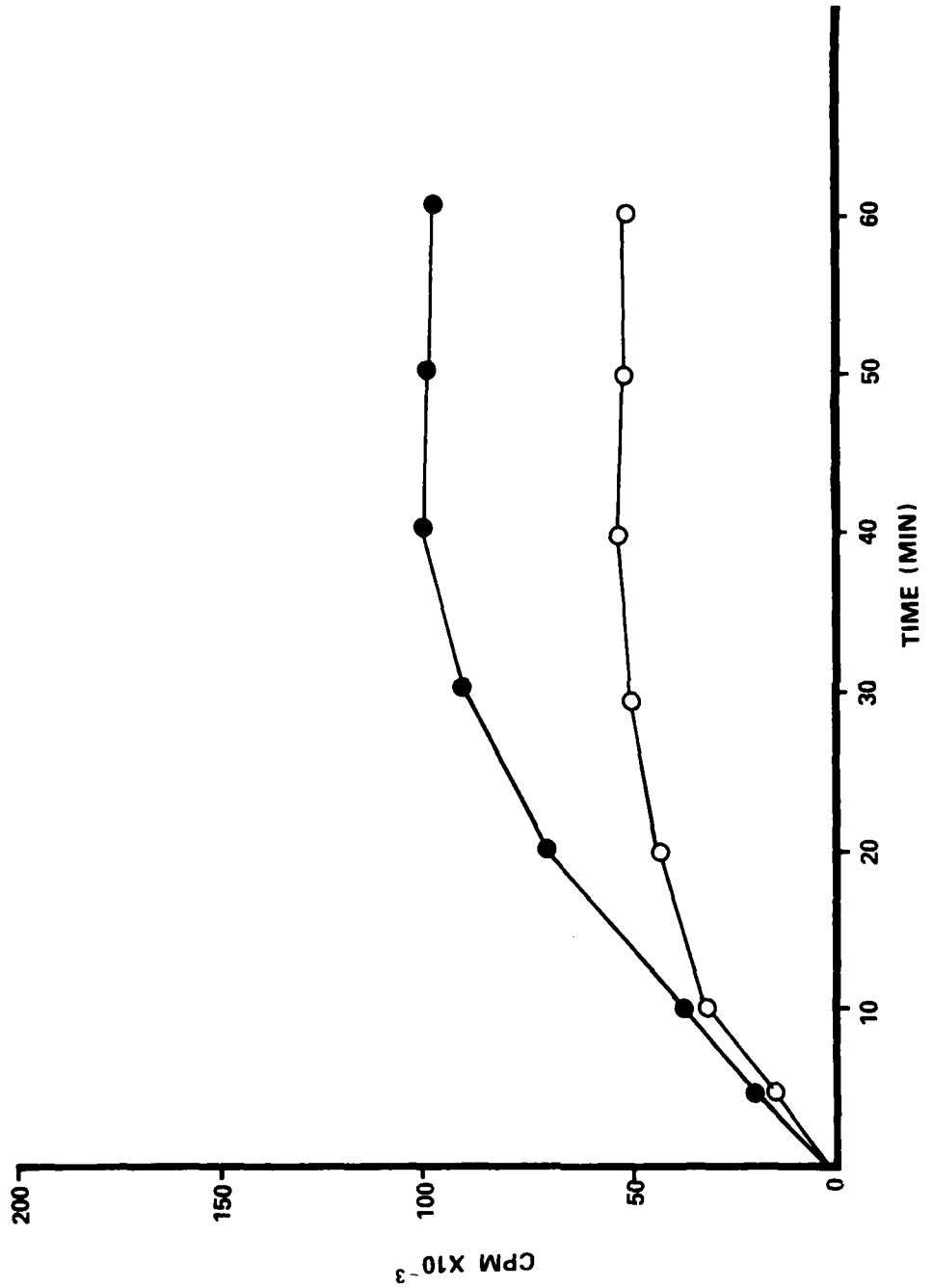

FIGURE 7

Chemiluminescence of bronchoalveolar cells lavaged from mice 24 hours after intratracheal inoculation of LPS. ○—○ control (20 μL saline); ●—● 20 μL of 5 μg/mL LPS.

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Chemiluminescence (CL) stimulated by the phagocytosis of yeast particles was used to monitor metabolic activity of murine macrophages obtained by either peritoneal or bronchoalveolar lavage. Intraperitoneal injection of the macrophage activator lipopolysaccharide (LPS) from Escherichia coli led to the appearance of macrophages in the peritoneal lavage fluid which had enhanced CL when compared to macrophages from control animals injected with saline or phosphate buffered saline. Intraperitoneal injection of LPS, however, was not able to enhance the CL activity of cells obtained by bronchoalveolar lavage whereas intratracheal instillation of LPS directly into the lung did elicit a population of cells with increased chemiluminescence. Thus it appears that CL is a useful measure of macrophage activation. The appropriateness of the CL technique was substantiated by the findings that enhanced CL induced by LPS is correlated to enhanced oxidative metabolism as measured by an increased production of hydrogen peroxide.

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Lipopolysaccharide

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